Title

Designing microenvironments for optimal outcomes in tissue engineering and regenerative medicine: From biopolymers to culturing conditions

Authors

Dimitrios Tsiapalis[*] (1, 2), Sofia Ribeiro* (1, 2, 3), Andrea De Pieri* (1, 2, 4), Ignacio Sallent* (1, 2), Salomé Guillaumin* (1, 2), Diana Gaspar (1, 2), Stefanie Korntner (1, 2), Yves Bayon (3), Manuela E. Gomes (5, 6, 7). Rui L. Reis (5, 6, 7), Dimitrios I. Zeugolis[†] (1, 2)

Affiliations

(1) Regenerative, Modular & Developmental Engineering Laboratory (REMODEL), Biomedical Sciences Building, National University of Ireland Galway (NUI Galway), Galway, Ireland

(2) Science Foundation Ireland (SFI) Centre for Research in Medical Devices (CÚRAM), Biomedical Sciences Building, National University of Ireland Galway (NUI Galway), Galway, Ireland

(3) Medtronic Sofradim Production, Trevoux, France

(4) Proxy Biomedical Ltd., Coilleach, Spiddal, Galway, Ireland

(5) I3Bs - Research Institute on Biomaterials, Biodegradables and Biomimetics, University of Minho, Portugal

(6) ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal

---

[*] Dimitrios Tsiapalis, Sofia Ribeiro, Andrea De Pieri, Ignacio Sallent and Salomé Guillaumin share first authorship.

[†] Corresponding Author: Dimitrios I. Zeugolis. Telephone: +353 (0) 9149 3166; Fax: +353 (0) 9156 3991; Email: dimitrios.zeugolis@nuigalway.ie

(7) The Discoveries Centre for Regenerative and Precision Medicine, Headquarters at University of Minho, Avepark, 4805-017 Barco, Guimarães, Portugal

Abstract

Bone marrow mesenchymal stem cells have been extensively used for tissue engineering and regenerative medicine applications due to their ease of isolation and expansion and their ability to differentiate towards various lineages of mesodermal origin. Despite these properties, their clinical potential is often hampered by the simplicity of the *in vitro* environment and its inability to resemble the complex *in vivo* niche. Herein, different microenvironmental cues (e.g. surface topography, substrate stiffness, mechanical stimulation, oxygen tension and co-culture systems) that have been utilised to enhance the therapeutic efficacy of bone marrow mesenchymal stem cells are discussed.

Keywords

Tissue engineering; Bone marrow mesenchymal stem cells; Microenvironmental cues; Surface topography; Substrate stiffness; Mechanical loading; Low oxygen tension; Co-culture systems

1. Introduction

Regenerative medicine is a rapidly evolving field that aims to functionally restore or replace tissues and organs. Tissue engineering approaches combine different cell types and materials in order to create functional tissue substitutes. The choice of cell source plays a paramount role in tissue engineering applications. Common requirements include a simple harvesting procedure, minimised donor site morbidity, high cell proliferation capacity, high cell expansion capacity without phenotypic drift. Mesenchymal stem cells (MSCs) fulfil these criteria, as they are adult stem cells with the ability to differentiate into mesodermal lineages, such as osteogenic, adipogenic, chondrogenic, neural, muscular, endothelial and tenogenic. MSCs can be isolated from various tissues, including bone marrow, adipose tissue, umbilical cord and peripheral blood. In particular, bone marrow-derived mesenchymal stem cells (BMSCs) have been extensively utilised due to their ease of isolation and their high expansion potential. BMSCs express stem cell specific surface markers, including STRO-1, CD29, CD73, CD90, CD105, CD146, Oct4 and SSEA4. In contrast, BMSCs are negative for the haematopoietic surface markers CD14 and CD34. Importantly, BMSCs express cytokines, such as TNF-α, TGF-β1 and IL-1β and display immunomodulatory properties by supressing the expansion and function of major immune cells, including dendritic cells, T cells, natural killer cells and macrophages. Although BMSCs are considered to be a promising cell type for various clinical applications, their efficacy is often hampered by the simplicity of the *in vitro* culture environment and its inability to recreate the complexity of their specific *in vivo* niche. Commonly utilised *in vitro* cell expansion protocols are based on growing stem cells on plastic surfaces that do not accurately imitate all important tissue-specific microenvironmental features, such as surface topography, substrate stiffness, mechanical stimulation, oxygen tension, localised density and interaction with other cell types (**Figure 1**).

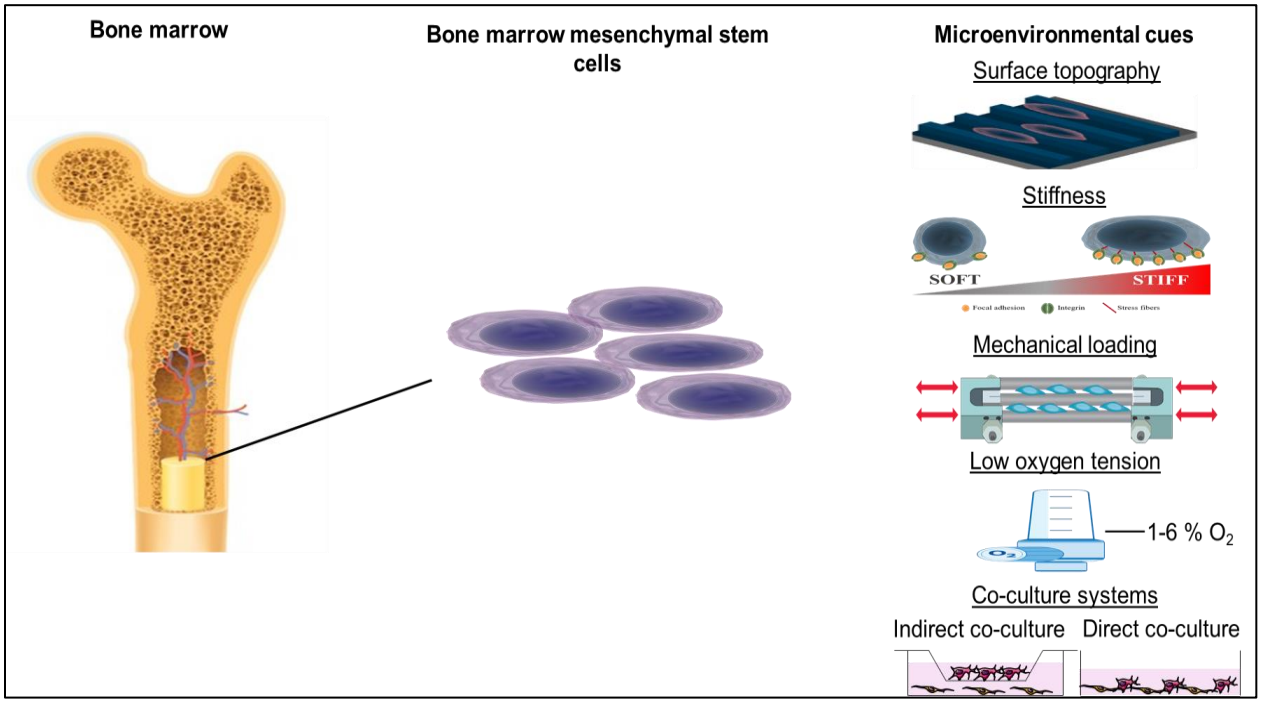

Figure 1: Different *in vitro* microenvironmental cues recapitulating the *in vivo* niche have been employed to modulate bone marrow mesenchymal stem cells behaviour.

There is a critical need to optimise *in vitro* culture conditions to fully unlock the therapeutic potential of BMSCs, either in their multipotent state or after guided differentiation towards the required lineage. Herein, we discuss current *in vitro* approaches based on microenvironmental cues, targeting the modulation of BMSC phenotype for improved therapeutic efficacy (summarised in **Table 1**).

Microenvironmental cues	Variable	Outcome
Surface topography	Electrospun aligned fibres	Cell alignment along the direction of fibre orientation
	Imprinted groves, nano-tubes, nano-pillars	Promotes osteogenic differentiation

Substrate Stiffness	Soft substrate	Differentiation towards neural or adipogenic lineage
	Hard substrate	Differentiation towards osteogenic lineage
Mechanical loading	Compressive loading	Promotes chondrogenic differentiation
	Hydraulic pressure	Increases gene expression of aggrecan, collagen type II and Sox9
	Shear stress	Differentiation into endothelial-like cells
	Tensile Forces	Increased expression of the osteogenic markers
Oxygen tension	Low oxygen tension (1 % – 6 %)	Increased BMSCs proliferation and migration Promote cell-fate commitment and cell differentiation towards mesodermal lineages (e.g. chondrogenic, osteogenic, neural, endothelial, tenogenic)
Co-culture systems	Direct co-culture of endothelial progenitor cells and BMSCs	Increased deposition of calcium with enhanced mineralization
	Indirect co-culture of tendon derived stem cells and tenocytes	Differentiation of BMSCs towards the tenogenic lineage

Table 1: Overview of the influence of the different *in vitro* microenvironmental cues on BMSCs behaviour.

2. Surface topography

Extensive studies have demonstrated that cell behaviour is significantly affected by the composition and surface topography of the culture substrate. Topographical cues are capable of directing stem cells with respect to proliferation, migration, differentiation and quiescence. Electrospinning (ES) is a technique often used to produce scaffolds that mimic ECM organisation. Due to its versatility and controllability ES represents a promising technique to manufacture fibrous three-dimensional scaffolds with controlled fibre diameter and alignment. As a bottom-up approach, it has only minimal processing waste and can be tailored to generate aligned nano- and micro- fibres with tuneable mechanical and structural features. Numerous natural and synthetic polymers have been used to fabricate electrospun scaffolds for tissue engineering applications targeting regeneration of bone, skin, cartilage, tendon and nerve, to mention only a few. Additionally, ES has been investigated for applications in drug delivery systems. It has been demonstrated that anisotropic nano-fibres guide cell alignment along the direction of fibre orientation. Scaffolds composed of aligned fibres enhanced the proliferation and differentiation of MSCs. Studies comparing random and aligned surface topographies, revealed that aligned poly (l-lactic acid) (PLLA) and polycaprolactone (PCL) electrospun scaffolds seeded with BMSCs induced cell orientation along the aligned fibres. Further, aligned scaffolds have promoted tenogenic differentiation of BMSCs through increased expression of tenogenic markers, such as scleraxis, mohawk, tenomodulin, tenascin-C and collagen type I. In more complex tissue engineering approaches, materials made of polyesters and natural polymers exhibiting non-organised topographies have been combined with biological cues. The synergistic effect of electrospun PLLA / gelatin matrices with randomly aligned fibres (diameter range 190-360 nm) and hepatogenic serum was shown to guide hepatic differentiation of BMSCs, demonstrating its potential as a hepatic substitute for restoring damaged liver function.

Surface patterning orientation has also been shown to affect neural differentiation of BMSCs. Random and aligned PCL fibres have been micropatterned with fibrin at different angles. A 45° angle promoted neurogenesis of BMSCs in basal media with a significant increase in neurogenic markers, such as microtubule-associated protein 2, nestin, neurogenic differentiation factor 1 and class III β-tubulin. To further assess cell-material interactions in depth, the influence of topographical cues and material chemistry was assessed by an extensive gene characterisation utilising RNA sequencing (RNA-Seq). BMSCs seeded on electrospun PCL fibres with a fibre diameter of 603 ± 197 nm showed that surface topography significantly altered gene expression more than the chemistry of the scaffold.

Although electrospinning has shown promising results, it offers limited control over topographical features. Lithography technologies have emerged from the demand for constructs with precise dimensional features such as grooves, pillars, pits and wells down to the nano-scale. One of the main advantages of imprint lithography is the accurate fabrication of topographies on a wide range of materials without altering their bulk properties. Soft lithography is the most commonly used, utilising elastomeric polymers to obtain patterns based on methods such as embossing, moulding and printing. The effect of topography on cell differentiation has been extensively studied using polymeric patterned substrates seeded with stem cells which have been differentiated towards chondrogenic, adipogenic, tenogenic and neural lineages. Advanced fabrication technologies have started elucidating the biological mechanisms that trigger a cell's morphological response to substrate topography. The conversion of filopodia into lamellipodia has shown to play an important role for cells responding to a material's topography. It has been shown that a microsphere array pattern promoted cell adhesion and proliferation of BMSCs due to accelerated lamellipodia formation and cell spreading with recognition and conversion of filopodia into lamellipodia. Further studies on BMSCs showed that micro-grooved topography affected primary cilia structure and function via the WNT signalling pathway. Further, substrate topography was shown to maintain stem cell multipotent phenotype, highlighting the potential

use for expansion prior to differentiation or transplantation. PCL substrates with a nanoscale pits have been shown to maintain BMSC phenotype and stem cell markers expression, such as STRO-1 and activated leukocyte cell adhesion molecule, CD166, up to eight weeks in basal conditions *in vitro*.

The differentiation ability of BMSCs is largely affected by cell adhesion, cell shape and size, elongation and position of focal adhesion points and cell-cell interactions, all of which indicate that surface topography will play a key role in control of stem cell lineage commitment. For instance, titanium substrates with different geometries, including groves, nano-tubes and nano-pillars have been shown to promote osteogenesis in BMSCs. Dots on PCL substrates with a diameter of 120 nm led to osteogenic differentiation of BMSCs, even in the absence of osteogenic media, with an increased activation of the extracellular signal-regulated kinases (ERK) - mitogen-activated protein (MAP) kinase cascade, which is crucial for osteogenic conversion. Polylactic-co-glycolic acid (PLGA) 85/15 films with varying width and spacing values of micropillars (between 0.8 and 6.4 μm values) were shown to induce severe deformation of cell nuclei. In the presence of lineage-specific differentiation media, osteogenesis was enhanced while adipogenesis was supressed. Polyesters, such as PCL, PLA and polyglycolide (PGA) were used to produce substrates with nano-pillar and nano-hole topographies, showing increased expression of chondrogenic markers, such as collagen II, aggrecan and proteoglycan 4 (PRG4) and enhanced formation of hyaline cartilage of BMSCs, whereas nano-grating resulted in insignificant chondrogenesis. Further, BMSCs seeded on thermoplastic polyurethane (TPU) with grid-like square cavities exhibited upregulated gene expression of anti-inflammatory markers involved in wound healing, indicating the potential of these topographical cues to enhance tissue regeneration.

Given the endless variations that can be introduced in the geometry of imprinted patterns, recent efforts are being directed towards developing high-throughput approaches for simultaneous assessment of multiple surfaces. A multi-patterned “biochip”, containing cues with sizes from 10 to 1000 nm, was explored in order to evaluate cellular-migration selectivity. BMSCs actively migrated towards the

patterns of preference, such as nano-grooves, while avoiding repelling topographies, such as nano-squared surfaces, as squares do not feature a topographical continuity necessary for cell migration. A similar approach has been developed to investigate the optimal topographies for osteogenic differentiation of BMSCs. Thousands of topographies were imprinted on a titanium-coated surface denominated TopoChip. Surfaces with highest alkaline phosphatase (ALP) expression have features of 10 to 30 μm with a moderate spacing of 5 to 10 μm. The cells were confined between the structures and were relatively narrow compared to cells on flat surfaces resembling a network of tubes. Additionally, these surfaces showed an upregulation of the osteogenic markers osteocalcin, osteopontin and bone sialoprotein. However, the TopoChip does not allow for a detailed investigation of the underlying mechanisms between stem cells and topographic cues. The TopoWellPlate, combining the previous described TopoChip and a 96 well plate, allows for the analysis of multiple genes and secreted proteins. Topographical cues are an important tool for control of *in vitro* BMSC culture. The use of optimal surface topographies for stem cell expansion is of particular interest when considering clinical translation of cell-based therapies. However, most approaches are focussed on surface patterning which does not allow for cell infiltration and does not provide the necessary three-dimensional architecture to accurately mimic the cells' native microenvironment. Discrepancies between *in vitro* and *in vivo* work have already been identified with regards to cell and tissue orientation. Further, the materials typically used frequently lack suitable mechanical properties and fail to emulate tissue stiffness in order to match the needs of BMSCs to commit to certain lineages.

3. Substrate stiffness

Adherent cells are known to respond to the elastic properties of their tissue-specific ECM, by adapting their cytoskeleton, initiating and coordinating signalling cascades. During embryonic development, matrix elasticity has been shown to be integral for driving cell differentiation. In adulthood, matrix

elasticity regulates cell-cycle progression and cell proliferation, making it an important factor in tissue homeostasis. Perturbations in ECM stiffness have been related to fibrosis, muscular dystrophies and malignant cancer progression. For decades, biological effects of matrix stiffness on *in vitro* cell cultures have been neglected by researchers with the generalised use of tissue culture plastic (TCP). Recent advances in material fabrication and characterisation technologies have allowed for the production of cell culture substrates with controlled surface rigidities, providing new insights into the role of this biophysical parameter on cell adhesion, migration, proliferation, morphology and gene expression.

Cells have been reported to sense their surrounding microenvironment via trans-membrane and cytoplasmic proteins that cluster together to form focal adhesion complexes (FAs). FAs act as bridges between the ECM and the cellular cytoskeleton, transmitting forces in an outside-in and inside-out fashion and serving as a key component in an incompletely understood force-sensitive signalling pathway, a process known as mechano-transduction. FAs and the actin cytoskeleton are dynamic structures; their size and degree of organisation directly correlates to the physical properties of the ECM. Stiff substrates provoke a spread-like cellular morphology with numerous FAs complexes and robust actin stress fibres. Conversely, soft substrates are known to induce a circular and constrained cellular morphology with immature FAs and disorganised actin filaments. Cytoskeletal tension is modulated by the activity of myosins, motor-proteins that slide actin filaments past one another. Active stretching of actin filaments results in a traction force from the cell towards the ECM which is proportional to the matrix rigidity. A phenomenon commonly known as durotaxis describes the differential cell migration guided by a rigidity gradient, typically from softer to stiffer substrates.

The elasticity of the ECM in humans ranges from a few Pascal (Pa) in soft tissues, such as brain, to GPa in hard tissues, such as bone. Several *in vitro* studies support the hypothesis that stem cells are able to recognise substrate stiffness and differentiate towards specific lineages. For instance, BMSCs cultured on soft (0.1 to 1 kPa), midrange compliant (8 to 17 kPa) and stiff (25 to 40 kPa) collagen type

I-coated polyacrylamide (PAAm) gels showed upregulated expression of neurogenic, myogenic and osteogenic markers, respectively. Similarly, soft fibronectin-coated PAAm gels (1 kPa) promoted adipogenic differentiation of BMSCs, while BMSCs seeded onto stiffer gels (40 to 68 kPa) underwent osteogenic differentiation. Other studies suggested that lineage commitment of stem cells is not dictated by matrix elasticity alone, but rather by a combination of matrix elasticity and other tissue-specific ECM-associated molecules. For example, BMSCs cultured on stiff PAAm substrates (80 kPa) showed significantly increased osteogenic differentiation potential when grown on collagen I coated gels. On gels coated with collagen IV and laminin I, two proteins marginally present in bone tissue, BMSCs did not exhibit osteogenic differentiation. Similarly, midrange compliant PAAm gels (15 kPa) alone failed to terminally differentiate BMSCs into smooth muscle cells. Instead, a combination of a specific substrate stiffness and transforming growth factor-beta (TGF-β) was required. In fact, TGF-β is widely known to promote BMSC differentiation towards two different types of phenotypes, depending on substrate compliance. In the presence of TGF-β, BMSCs cultured on stiff TCP adopted a smooth muscle cell (SMC)-like phenotype, whilst BMSCs cultured on hydrogels were directed towards a chondrogenic phenotype. It is noteworthy that some of the FA-proteins involved in mechano-transduction pathways, such as focal-adhesion kinases (FAK) and Src family kinases, are also key regulators in growth-factor-mediated signalling cascades. Collectively, matrix stiffness as physical cues work in concert with other biological and biochemical signals, influencing stem cell lineage commitment.

Despite encouraging results, restricted availability of stiffness-tuneable biodegradable materials, lack of standardised procedures for the measurement of substrate rigidities and insufficient knowledge of the native stiffness of most human tissues pose important limitations for successful research in this field. Despite natural polymers like collagen, gelatin and fibrin are readily implantable and present important cell binding domains, they can only be modulated in order to display a small range of soft rigidities.

The most commonly used materials for stiffness-related studies are polyacrylamide and polydimethylsiloxane (PDMS), two synthetic polymers that can be easily cross-linked to achieve an extensive range of physiologically relevant stiffness. However, the cytotoxicity of the former and the non-biodegradability of the latter hinder their use as implantable devices and restrict their application as *in vitro* cell culture surfaces. Therefore, novel biomaterials or fabrication technologies are imperative for future advances in the field. Further, material stiffness is not only dependent on its intrinsic mechanical properties, but also on the methodology chosen to measure it. Stiffness measurements performed with different techniques (indentation, rheometry, tensile and compression testing) and under different testing conditions (temperature, hydration of the material, tensile and compressive test strain rates) result in different Young's modulus values for the same material sample. Although atomic force microscopy (AFM) indentation is considered the most accurate technique to measure the matrix micro-compliance sensed by cells, different techniques used across the literature lead to misconceptions. Ultimately, comprehensive studies on human tissue rigidities that would strongly potentiate the design of biomimetic materials remain elusive.

In summary, matrix rigidity offers great promise as a biophysical tool for controlling stem cell phenotype and differentiation. Its use for *in vitro* cell culture substrates could potentially overrule phenotypic drift of multipotent stem cells associated with long-term cultures, facilitating cell expansion for cellular therapies. Precise substrate stiffness cues incorporated into smart scaffolds potentially driving lineage-specific stem cell differentiation, improving tissue engineering approaches for implantable devices.

4. Mechanical loading

Myriad forces regulate tissue physiology and homeostasis in living bodies. Commonly, they operate in form of compressive loading, hydraulic pressure, shear stress and tensile forces. Differentiated cells,

such as endothelial cells, fibroblasts, osteocytes, tenocytes chondrocytes and even stem cells are mechano-sensitive, constituting key players in the body's responses to mechanical forces. Cells can react to mechanical stimuli through conformational or organisational changes in cellular molecules, such as integrins at focal adhesions, cadherin complexes in cell–cell adhesions and mechanosensitive ion channels. Thus, they regulate signalling pathways involved in cell growth, differentiation, cell survival or programmed cell death. Hence, for *in vitro* engineering of functional tissues, it is essential to mimic their mechanical *in vivo* microenvironment. Additionally, mechanical stimuli are crucial for the regulation of stem cell behaviour by influencing cell proliferation, self-renewal capacity and differentiation. Externally applied forces have been extensively described to modulate BMSC lineage commitment. For example, BMSCs seeded on non-woven scaffolds differentiated into endothelial-like cells, expressing markers such as van Willebrand factor (vWF), CD31, and laminin, after physiological shear stress stimulation. Further, many studies have reported that mechanical stress promoted osteogenic differentiation of BMSCs. Stimulation of BMSCs with cyclic tensile stretch for 6 hours led to an increased expression of the osteogenic markers osteocalcin and Runx2. Similarly, mechanical stretch facilitated osteogenesis in human jaw bone marrow mesenchymal stem cells by blocking nuclear factor-kB activity. Further, mechanical loading promoted osteogenic differentiation of goat BMSCs cultured on PLGA scaffolds. Accumulating evidence increasingly suggests that mechanical loading positively affects tenogenic differentiation of BMSCs. In one study, human jaw bone marrow BMSCs were subjected to cyclical uniaxial stretching of 4 %, 8 % and 12 % strain. A strain of 8 % led to increased collagen production and expression of the tendon-associated markers tenascin-C, scleraxis and tenomodulin, mediated by stretch-activated calcium channels. However, a similar study considered 10 % strain as optimal to induce tenogenic differentiation of BMSCs. Both, gene expression and protein levels were strongly correlated with cell orientation. Finally, in a dynamic 3D model, tenogenic differentiation of BMSCs was mediated by Wnt4/Wnt5 signalling.

Mechanically-generated signals also showed effects on the chondrogenic differentiation potential of BMSCs. Regarding cartilage tissue engineering and mechanobiology, so far, dynamic compressive loading has been one of the most utilised model systems for mechanical stimulation. Dynamic loading of BMSC-laden constructs increased aggrecan promoter activity and accumulation of sulphated glycosaminoglycans (sGAG). The combination of growth factor supplementation, especially TGF-β1, and mechanical loading increased gene expression of aggrecan and collagen type II. Further, intermittent hydrostatic pressure has been reported to increase gene expression of aggrecan, collagen type II and Sox9 in human BMSCs, compared to untreated controls in the absence of TGF-β1. Multifactorial approaches involving the combination of shear and dynamic compression have been shown to increase gene expression of chondrogenic markers, gene expression of TGF-β1 and TGF-β3 and protein synthesis in BMSCs. In addition, studies targeting muscle tissue regeneration have assessed the potential of BMSCs to differentiate into skeletal muscle cells. Uniaxial cyclic loading initiated myogenic differentiation without the use of growth factors by increasing mRNA levels of myogenic regulatory factors MyoD and MyoG. Furthermore, the combination of uniaxial loading and insulin-like growth factor 1 (IGF-1) resulted in increased expression of myogenic markers. Similarly, cyclic stretch caused BMSCs to differentiate into smooth muscle cells, by directing fibre alignment and enhancing α-smooth muscle actin expression.

Bioreactors represent one of the key technologies developed in order to induce mechanical stimuli *in vitro*. The use of bioreactors allows for the implementation of mechanical stimulation and concomitant regulation of nutrient and gas exchange during the whole culture period, potentially contributing to the maturation of functional tissue substitutes *in vitro*. In recent years, more sophisticated technologies have been developed in order to precisely recreate physiological microenvironments *in vitro*. Among them, organ-on-a-chip technologies gained considerable success by recapitulating multicellular architectures, physicochemical microenvironments and vascular perfusion. By closely imitating

physiological conditions, these systems allow for assessing synergistic effects of stiffness, strain, shear forces and additional features in a high throughput format, potentially directing stem cell differentiation more precisely.

A significant number of studies demonstrated the value of mechanical loading for modulating BMSC differentiation *in vitro*. However, further studies need to investigate the underlying mechanisms by unravelling mechanosensitive pathways involved in BMSC lineage commitment.

5. Oxygen tension

Oxygen tension is considered to be an integral component regulating developmental processes, cell fate and tissue function. *In vivo*, tissues experience a wide range of oxygen tensions, depending on their location and capillary supply, which are notably different from the inhaled oxygen tension of ~20 %. The levels of oxygen pressure steadily decrease after entering the lungs and travelling in the bloodstream through the body. Having reached the respective organs, oxygen levels have dropped to approximately 2 % to 9 %. Generally, BMSC-niches are located in regions of low oxygen tension, ranging from 1 % to 6 %. Recent evidence has identified hypoxia to contribute to maintaining a stable phenotype and undifferentiated state of BMSCs. Moreover, various levels of low oxygen tension (1 % – 6 %) are known to affect cell proliferation, cell-fate commitment and cell differentiation, frequently in combination with other microenvironmental cues. Therefore, considerable research effort has been conducted in order to investigate a wide range of oxygen tensions with respect to different tissue engineering applications.

Oxygen tension at low, physiological ranges has been shown to affect the proliferation rates of different cell types. A plethora of studies have highlighted the beneficial effects of hypoxia-inducible factor (HIF-1α), which is activated under low oxygen conditions, on proliferation and expansion rates of BMSCs. HIF-1α enhanced BMSC-proliferation through the stimulation of Twist-related protein, which

in turn downregulated the cyclin-dependent kinase inhibitor 1 (p21) and increased proliferation, thereby bypassing cell senescence. Activation of HIF-1α at 1 % and 5 % oxygen tension promoted the expression of the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) and downregulated the pro-apoptotic proteins Bcl-2-associated X protein (BAX) and cleaved caspase-3 in rat BMSCs *in vitro*. Furthermore, HIF-1α upregulated the ratio of phosphorylated extracellular-signal regulated kinase 1/2 (ERK), which plays a significant role in intracellular signalling for cellular proliferation and survival. Similarly, 1 % oxygen tension inhibits alterations in cell morphology and cell size and delayed the expression of senescence-associated β-galactosidase, retaining the expression of multipotency markers and chemokine-related genes, such as OCT4 and C-X-C chemokine receptor type 7 (CXCR7). Nevertheless, preconditioning of BMSCs under hypoxia in the presence of fibroblast growth factor (FGF) - 2 increased cell proliferation and migration rates, while affecting multipotency by increasing chondrogenic and osteogenic differentiation. One study investigated porcine BMSCs in two- and three-dimensional culture systems under 2 % oxygen tension. After 40 days of culture, BMSC proliferation was increased; however, the osteogenic differentiation potential was reduced, when compared with cells cultured under 20 % oxygen tension conditions.

A rationale of using BMSCs for tissue engineering approaches is their ability to migrate to the site of tissue damage. HIF-1α promotes the expression of CXCR4 and CX3CR1 in BMSCs, which stimulate cell migration and engraftment after transplantation. Similarly, hypoxia increased phosphorylation of cell migration related proteins c-Jun N-terminal kinases (JNK), focal adhesion kinase (FAK) and ERK1/2, as well as signal transducer and activator of transcription 3 (STAT3). Moreover, hypoxia-activated HIF-1α significantly increased BMSCs migration via downregulation of integrin α4 and upregulation of Rho associated kinase ROCK1 and serine/threonine kinase (Rac1/2/3) pathways. Stemness is characterised by the ability of stem cells to self-renew and to differentiate into multiple lineages, which is a prerequisite for cell-based therapies. Under ambient oxygen tension, BMSCs have

shown to undergo senescence, whereas hypoxic conditions (1 %) have promoted multipotency, which was attributed to the downregulation of p16 expression. Moreover, subjecting BMSCs to 5 % oxygen tension during expansion from passage 0 (p0) up to passage 3 (p3) preserved an undifferentiated and multipotent state. Additionally, cells subjected to 5 % hypoxia contained less mitochondria and exhibited an undifferentiated morphology, compared to cells grown under normoxic conditions. Furthermore, 2 % oxygen tension in combination with macromolecular crowding, a biophysical phenomenon known to accelerate extracellular matrix deposition, resulted in a microenvironment capable of maintaining the phenotype of BMSCs and their multilineage potential. In summary, low oxygen tension is of great importance for maintaining BMSC plasticity.

With regards to multilineage differentiation potential of BMSCs, several studies have compared hypoxic preconditioning of cells versus a continued hypoxic culture. Interestingly, chondrogenesis was promoted when BMSCs were isolated and expanded at an oxygen tension of 3 %, compared to cells cultured in normoxia. Similarly, an increased chondrogenic potential of ovine BMSCs was observed after isolation, expansion and differentiation in hypoxia (3 %), when seeded on collagen and hyaluronan (HA) scaffolds. Another study illustrated elevated chondrogenic marker expression when BMSCs were cultured at a low oxygen tension (5 %); however, this effect was attributed to the type of scaffold used. Thus, 5 % oxygen tension was able to induce chondrogenesis of BMSCs grown on PCL, HA and on collagen type I scaffolds. Additionally, BMSCs cultured at 2 % oxygen tension with chondrogenic induction media, showed that increased expression of HIF-1*a* led to phosphorylation of both protein kinase B and mitogen activated protein kinase p38, which in turn resulted in an upregulation of chondrogenic markers (collagen type II, Sox-9) and increased proteoglycan deposition. The combination of 3 % oxygen tension, collagen scaffolds, and bone morphogenetic protein 2 (BMP-2) and TGF-ss1 supplementation strongly increased chondrogenic differentiation of equine BMSCs,

compared to normoxic control conditions, resulting in the *in vitro* synthesis of hyaline-like neocartilage.

Hypoxia has been shown to also play a role in osteogenic differentiation of BMSCs. Hypoxic (1 %) preconditioning of BMSCs enhanced both osteogenesis and chondrogenesis *in vitro*, while it promoted osteogenesis in an *in vivo* mouse ectopic model. Furthermore, hypoxia enhanced the formation of a stable ECM *in vivo*, as revealed by the increased soluble and insoluble collagen production and collagen type I and III expression. In another study, expansion of BMSCs under low oxygen tension (5 %) promoted osteogenesis. Similarly, 5 % oxygen tension was found to promote osteogenic and angiogenic responses of BMSCs more effectively compared to cells in normoxia, when cultured on bone-derived scaffolds. These effects were attributed to the activation of the ERK1/2 and p38 pathway, induced by a preceding HIF-1α activation. Arg-Gly-Asp (RGD peptides) incorporated into biomaterials have previously been shown to upregulate osteoblastic differentiation in MSCs. Interestingly, stabilisation of HIF-1α resulted in an enhanced osteogenic and angiogenic potential of BMSCs seeded in RGD hydrogels after low oxygen preconditioning. Additionally, reduced osteogenic and adipogenic differentiation of BMSCs was observed in a low oxygen culture (1 %) supplemented with platelet lysate. Interestingly, increasing the oxygen tension to 3 % allowed for a recovery of the cells' osteogenic potential. In addition, the effects of hypoxia have been studied in different osteonecrosis models. Stem cells extracted from the bone marrow of osteonecrotic rabbits exhibited a decreased proliferation ability, loss of multipotency, reduced osteoblastic differentiation and increased adipogenic potential. Notably, when exposed to a hypoxic environment, extracted BMSCs showed enhanced proliferation and osteogenic potential, highlighting a beneficial effect of hypoxia for osteonecrosis-related therapies. Besides chondrogenesis and osteogenesis, different studies explored the influence of low oxygen tension on the differentiation of BMSCs towards other mesenchymal lineages. Hence, combined low oxygen tension (2 %) and endothelial growth medium were used in order to stimulate

endothelial differentiation. Further, 1 % oxygen tension combined with epidermal growth factor (EGF) and basic fibroblast growth factor supplementation was used to generate neural progenitors. In another study, transplantation of hypoxic preconditioning BMSCs into rat Achilles tendon defects improved the healing outcome, compared to normoxic BMSCs.

Culture of BMSCs under physiologically low oxygen tensions has shown significant benefits with respect to cell proliferation, migration, plasticity and differentiation. However, the ideal combination of microenvironmental cues for controlling stem cell phenotype and differentiation still remain unclear and new approaches need to be explored in order to more closely mimic the native *in vivo* microenvironment of the respective tissues.

6. Co-culture systems

The human body comprises a complexity of multiple, distinct cell types which are in charge of different functions. Due to this heterogeneity, cells interact and communicate closely with each other. The combination of different cell populations using *in vitro* co-culture systems allows for a closer recapitulation of the native *in vivo* microenvironment with the aim to direct *in vitro* stem cell behaviour more accurately. Different methodologies such as direct and indirect co-cultures are used in tissue engineering approaches. In direct co-culture systems the different cell types are in direct contact with each other. This allows for direct cell-cell interactions via gap-junctions and ECM. Distinct cell populations are separated by a permeable membrane and cell interactions occur exclusively via paracrine secretion. Within the emerging stem cell field, co-culture has rapidly become a suitable tool for controlling differentiation, proliferation and phenotype more effectively. A recent review discussed co-culture systems for different tissue engineering applications in detail, with covering tissues such as cartilage, ligament, bone, heart, liver, lung and kidney. Co-culture for bone tissue engineering focusses on inducing vascularisation of the bone constructs, a crucial element for efficient bone regeneration.

Bone morphogenetic proteins (BMPs) have been reported to play a key role in osteogenesis. In one study, BMP-2 facilitated the osteogenic differentiation of BMSCs by upregulating the expression of alkaline phosphatase (ALP) and osteocalcin. More recently, efforts have been directed towards more complex co-culture systems utilising scaffolds or bioreactors. One study reported the formation of an osteoid, when BMSCs and endothelial progenitor cells were co-seeded on a polysaccharide scaffold comprised to pullulan and dextran. In another study, BMSC-derived endothelial cells and BMSCs were co-cultured on tricalcium phosphate scaffolds and transplanted into large segmental bone defects in rabbits. A highly vascularised tissue with improved mechanical properties proved the effectiveness of this co-culture approach for bone tissue engineering. When endothelial progenitor cells derived from umbilical cord blood were co-cultured with BMSCs and subjected to mechanical stimuli in a bioreactor, an increased deposition of calcium with enhanced overall mineralization and vessel infiltration of the constructs were reported.

The discovery of the chondrogenic potential of BMSCs lead to studies investigating co-cultures of chondrocytes and BMSCs. Both in direct and indirect configurations, chondrocyte proliferation was increased and the presence of chondrocytes induced chondrogenic differentiation of BMSCs. Similar results were obtained in three-dimensional co-culture systems using PCL microfibre mats. Additionally, co-culture with synovial fluid or synovial cells triggered chondrogenic differentiation of BMSCs. Generally, a dense ECM is responsible for the specific biomechanical properties of cartilage. Co-culture of meniscus cells and BMSCs under low oxygen tension promoted a cartilage-specific ECM formation with increased expression of aggrecan, collagen type I and collagen type II. The development of bioreactors has contributed to the development of various co-culture approaches for cartilage tissue regeneration. In one study, a dynamic bioreactor induced the spontaneous formation of 3D aggregates of articular chondrocytes when co-cultured with BMSCs. A lower mRNA ratio of

collagen type I / collagen type II was achieved and glycosaminoglycan contents increased more than 2 fold compared to single cultures.

Co-culture studies targeting ligament tissue engineering mainly involve BMSCs and anterior cruciate ligament (ACL) cells in direct and indirect systems. Mechanical loading is essential for ligament tissue engineering, for this reason, studies applied various mechanical loading regimes in direct and indirect co-culture systems. In an indirect co-culture approach, using a trans-well system, ligament cells were seeded in the lower chamber, whereas a gelatine / silk-fibroin scaffold seeded with BMSCs was positioned in the trans-well. Thus, differentiation of BMSC towards ligament-like cells was achieved. Studies demonstrated successful differentiation of BMSCs towards the tenogenic lineage and increased cell proliferation by direct co-culture with tendon derived stem cells (TDSCs), by indirect co-culture with tenocytes and by culturing BMSCs in the presence of tendon tissue fragments. Co-culture of BMSCs and TDSCs enabled the formation of cell sheets, that significantly promoted tendon healing in a rat patellar tendon window defect model, compared to cell sheets generated with a single-cell type. Co-cultured cell-sheets lead to an improved alignment of collagen fibres with more elongated cells and tendons exhibited a higher ultimate load to failure and Young's modulus.

BMSCs have previously been shown to play a pivotal role in cardiac regeneration. In a 3D co-culture system BMSCs were co-seeded with ventricular embryonic cardiomyocyte. BMSCs differentiated into cardiomyocyte-like cells that exhibited spontaneous contraction. Co-culture of BMSCs and transformed lung epithelial cells has been performed in order to direct differentiation towards pulmonary cells. BMSCs in co-culture expressed epithelial markers specific for lung tissue such as cytokeratin 5, 8, 14, 18, 19, pro-surfactant protein C and zonula occludens-1 (ZO1). BMSCs co-cultured with proximal tubular epithelial cells led to improved cell viability and proliferation of the latter cell population. Further, co-culture approaches were used in order to investigate cytoplasm and organelle transfer between different cell types. When BMSCs were directly co-cultured with renal

tubular cells, the formation of intercellular contacts, such as tunnelling nanotubes, was observed. Using fluorescent probes specific to mitochondria, cytosol, plasmalemma, transport of cellular contents through nanotubes was observed in both directions, direct and retrograde. Successful differentiation of BMSCs into renal tubular cells was attributed to this exchange of contents. In tissue engineering applications targeting liver regeneration, direct co-culture of hepatocytes and BMSCs resulted in hepatogenic differentiation and formation of three-dimensional liver spheroids. Further, beneficial effects such as preservation of hepatocyte morphology, improved cell viability and increased ECM deposition was observed in co-cultures of hepatocytes and BMSCs. In a similar approach, serum derived from rats with acute liver failure (ALF) induced secretion of anti-inflammatory molecules when added to co-culture systems, caused by changes in the BMSC secretome. Compared to single cell sources and other co-cultures, liver assisted devices (LAD) containing cocultures of BMSCs and hepatocytes showed the highest cell survival, indicating its potential use for therapies targeting acute liver failure. Liver fibrosis is partially caused by the activation and proliferation of hepatic stellate cells and an increase in ECM deposition. In one study, co-culturing BMSCs and hepatic stellate cells led to a decrease of ECM deposition and an inhibition of hepatic stellate cell activation by BMSCs, posing a potential application to prevent liver fibrosis.

Co-culture systems, in both direct and indirect configurations, have shown great potential for various clinical targets regarding tissue engineering and regeneration. These systems aim to recapitulate the native *in vivo* microenvironment more accurately and therefore contribute to a deeper understanding of cell-cell and cell-tissue interactions. Further, co-cultures pose valuable tools for the development of novel *in vitro* models and tissue engineering strategies.

7. Conclusions

Conventional *in vitro* culture systems fail to imitate native microenvironments of the respective tissues, compromising stem cell viability and proliferation, finally leading to cellular senescence, loss of multipotency and phenotypic drift. The ability of BMSCs to self-renew and differentiate into various lineages has been extensively investigated in emerging fields such as tissue engineering, gene therapy and regenerative medicine. However, the control of stem cell fate *in vitro* and *in vivo* due to the manifold underlying processes is yet to be fully understood. This review emphasises novel *in vitro* approaches employing different microenvironmental cues, such as surface topography, substrate stiffness, mechanical loading, oxygen tension and co-culture, aiming to elucidate the underlying mechanisms involved in cell-substrate interactions.

Other *in vitro* microenvironment modulators, such as macromolecular crowding, which has been shown to enhance ECM deposition and to enable stable expansion of embryonic stem cells are expected to play a pivotal role in stem cell expansion and in accelerated development of tissue equivalents in the years to come. Even though the use of single cues showed promising results, the integration of multiple cues within a system is a relatively unexplored field. It is expected that the combination of different cues and their synergistic effects contribute to a more accurate recapitulation of the complex *in vivo* niche. Moreover, an improved understanding of direct and indirect mechanisms regulating BMSC phenotype maintenance and differentiation will pave the way for novel methods and tools in the fields of tissue engineering and regenerative medicine.

8. Acknowledgements

The authors would like to acknowledge the following entities for financial support: H2020, Marie Skłodowska-Curie Actions, Innovative Training Networks 2015 Tendon Therapy Train project (Grant No. 676338); Science Foundation Ireland (SFI) / European Regional Development Fund (Grant Number 13/RC/2073); and SFI Career Development Award (Grant Number 15/CDA/3629).

9. Relevant literature

1. Vacanti, J.P. and R. Langer, *Tissue engineering: the design and fabrication of living replacement devices for surgical reconstruction and transplantation.* Lancet, 1999. **354 Suppl 1**: p. Si32-4.
2. Howard, D., et al., *Tissue engineering: strategies, stem cells and scaffolds.* Journal of Anatomy, 2008. **213**(1): p. 66-72.
3. Polymeri, A., W.V. Giannobile, and D. Kaigler, *Bone marrow stromal stem cells in tissue engineering and regenerative medicine.* Hormone and Metabolic Research, 2016. **48**(11): p. 700-713.
4. Wang, C., et al., *Differentiation of Bone Marrow Mesenchymal Stem Cells in Osteoblasts and Adipocytes and its Role in Treatment of Osteoporosis.* Medical Science Monitor: International Medical Journal of Experimental and Clinical Research, 2016. **22**: p. 226-233.
5. Zheng, Y.H., et al., *Multilineage differentiation of human bone marrow mesenchymal stem cells in vitro and in vivo.* Experimental and Therapeutic Medicine, 2013. **5**(6): p. 1576-1580.
6. Solchaga, L.A., K.J. Penick, and J.F. Welter, *Chondrogenic differentiation of bone marrow-derived mesenchymal stem cells: tips and tricks.* Methods in Molecular Biology, 2011. **698**: p. 253-78.
7. Galli, D., M. Vitale, and M. Vaccarezza, *Bone marrow-derived mesenchymal cell differentiation toward myogenic lineages: Facts and perspectives.* BioMed Research International, 2014. **2014**: p. 762695.
8. Liu, W., et al., *Directing the differentiation of parthenogenetic stem cells into tenocytes for tissue-engineered tendon regeneration.* Stem Cells Translational Medicine, 2017. **6**(1): p. 196-208.

9. Fraser, J.K., et al., *Plasticity of human adipose stem cells toward endothelial cells and cardiomyocytes.* Nature Clinical Practice Cardiovascular Medicine, 2006. **3**(1): p. S33-7.

10. Hass, R., et al., *Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC.* Cell Communication and Signaling, 2011. **9**: p. 12-12.

11. Gang, E.J., et al., *SSEA-4 identifies mesenchymal stem cells from bone marrow.* Blood, 2007. **109**(4): p. 1743-51.

12. Greco, S.J., K. Liu, and P. Rameshwar, *Functional similarities among genes regulated by OCT4 in human mesenchymal and embryonic stem cells.* Stem Cells, 2007. **25**(12): p. 3143-54.

13. Haynesworth, S.E., M.A. Baber, and A.I. Caplan, *Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies.* Bone, 1992. **13**(1): p. 69-80.

14. Sacchetti, B., et al., *Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment.* Cell, 2007. **131**(2): p. 324-36.

15. Conget, P.A. and J.J. Minguell, *Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells.* Journal of Cell Physiology, 1999. **181**(1): p. 67-73.

16. Uccelli, A., L. Moretta, and V. Pistoia, *Mesenchymal stem cells in health and disease.* Nature Reviews Immunology, 2008. **8**(9): p. 726-36.

17. Uccelli, A., V. Pistoia, and L. Moretta, *Mesenchymal stem cells: a new strategy for immunosuppression?* Trends in Immunology, 2007. **28**(5): p. 219-26.

18. Heitman, N., N. Saxena, and M. Rendl, *Advancing insights into stem cell niche complexities with next-generation technologies.* Current Opinion in Cell Biology, 2018. **55**: p. 87-95.

19. Guillaumin, S., I. Sallent, and D.I. Zeugolis, *Biophysics rules the cell culture but has yet to reach the clinic: Why is that?* Journal of the American Academy of Orthopaedic Surgeons, 2017. **25**(7): p. e144-e147.

20. Cigognini, D., et al., *Engineering in vitro microenvironments for cell based therapies and drug discovery.* Drug Discovery Today, 2013. **18**(21-22): p. 1099-1108.

21. Jiang, J. and E.T. Papoutsakis, *Stem-cell niche based comparative analysis of chemical and nano-mechanical material properties impacting ex vivo expansion and differentiation of hematopoietic and mesenchymal stem cells.* Advanced Healthcare Materials, 2013. **2**(1): p. 25-42.

22. Nikkhah, M., et al., *Engineering microscale topographies to control the cell-substrate interface.* Biomaterials, 2012. **33**(21): p. 5230-46.

23. Klymov, A., et al., *Understanding the role of nano-topography on the surface of a bone-implant.* Biomaterials Science, 2013. **1**(2): p. 135-151.

24. Fernandez-Yague, M.A., et al., *Biomimetic approaches in bone tissue engineering: Integrating biological and physicomechanical strategies.* Advanced Drug Delivery Reviews, 2015. **84**: p. 1-29.

25. Spanoudes, K., et al., *The biophysical, biochemical, and biological toolbox for tenogenic phenotype maintenance in vitro.* Trends in Biotechnology, 2014. **32**(9): p. 474-482.

26. Ballester-Beltrán, J., et al., *Sensing the difference: The influence of anisotropic cues on cell behavior.* Frontiers in Materials, 2015. **2**.

27. Dalby, M.J., et al., *Nanotopographical control of human osteoprogenitor differentiation.* Current Stem Cell Research & Therapy, 2007. **2**(2): p. 129-138.

28. Biggs, M., A. Pandit, and D.I. Zeugolis, *Imprinting lithography and electrospinning are due to transform healthcare.* Nanomedicine, 2016. **11**(9): p. 989-992.

29. Lomas, A., et al., *The past, present and future in scaffold-based tendon treatments.* Advanced Drug Delivery Reviews, 2015. **84**: p. 257-277.

30. Fuller, K., P. A., and Z.D. I., *The multifaceted potential of electro-spinning in regenerative medicine.* Pharmaceutical Nanotechnology, 2014. **2**(1): p. 23-34.

31. Braghirolli, D.I., D. Steffens, and P. Pranke, *Electrospinning for regenerative medicine: a review of the main topics.* Drug Discovery Today, 2014. **19**(6): p. 743-753.

32. Rogina, A., *Electrospinning process: Versatile preparation method for biodegradable and natural polymers and biocomposite systems applied in tissue engineering and drug delivery.* Applied Surface Science, 2014. **296**: p. 221-230.

33. Repanas, A. and G. Birgit, *The significance of electrospinning as a method to create fibrous scaffolds for biomedical engineering and drug delivery applications.* Journal of Drug Delivery Science and Technology, 2016. **31**: p. 137-146.

34. Kai, D., et al., *Electrospun synthetic and natural nanofibers for regenerative medicine and stem cells.* Biotechnology Journal, 2013. **8**: p. 59-72.

35. Zeugolis, D.I., et al., *Electro-spinning of pure collagen nano-fibres - just an expensive way to make gelatin?* Biomaterials, 2008. **29**(15): p. 2293-305.

36. Yoshimoto, H., et al., *A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering.* Biomaterials, 2003. **24**(12): p. 2077--2082.

37. Chang, J.C., et al., *Cell orientation and regulation of cell-cell communication in human mesenchymal stem cells on different patterns of electrospun fibers.* Biomedical Materials, 2013. **8**(5): p. 055002.

38. Hodgkinson, T., Y. Xue-Feng, and B. Ardeshir, *Electrospun silk fibroin fiber diameter influences in vitro dermal fibroblast behavior and promotes healing of ex vivo wound models.* Journal of Tissue Engineering, 2014. **5**: p. 1-13.

39. Garrigues, N.W., et al., *Electrospun cartilage-derived matrix scaffolds for cartilage tissue engineering.* Journal of Biomedical Materials Research Part A, 2014. **102**(11): p. 3998-4008.

40. Wise, J.K., et al., *Chondrogenic differentiation of human mesenchymal stem cells on oriented nanofibrous scaffolds: Engineering the superficial zone of articular cartilage.* Tissue Engineering, 2009. **15**(4): p. 913-921.

41. Sahoo, S., et al., *Characterization of a novel polymeric scaffold for potential application in tendon/ligament tissue engineering.* Tissue Engineering, 2006. **12**(1): p. 91--99.

42. Schnell, E., et al., *Guidance of glial cell migration and axonal growth on electrospun nanofibers of poly-e-caprolactone and a collagen/poly-e-caprolactone blend.* Biomaterials, 2007. **28**(19): p. 3012--3025.

43. Khorshidi, S., et al., *A review of key challenges of electrospun scaffolds for tissue-engineering applications.* Journal of Tissue Engineering and Regenerative Medicine, 2016. **10**(9): p. 715-38.

44. Ma, J., X. He, and E. Jabbari, *Osteogenic differentiation of marrow stromal cells on random and aligned electrospun poly(L-lactide) nanofibers.* Annals of Biomedical Engineering, 2011. **39**(1): p. 14-25.

45. Martins, A., et al., *The influence of patterned nanofiber meshes on human mesenchymal stem cell osteogenesis.* Macromolecular Bioscience, 2011. **11**(7): p. 978-87.

46. Rothrauff, B.B., et al., *Braided and stacked electrospun nanofibrous scaffolds for tendon and ligament tissue engineering.* Tissue Engineering, 2017. **23**(9-10): p. 378-389.

47. Barber, J.G., et al., *Braided nanofibrous scaffold for tendon and ligament tissue engineering.* Tissue Engineering, 2013. **19**: p. 1265-1274.

48. Bishi, D.K., et al., *A patient-inspired ex vivo liver tissue engineering approach with autologous mesenchymal stem cells and hepatogenic serum.* Advanced Healthcare Materials, 2016. **5**(9): p. 1058-1070.

49. Li, H., et al., *Micropatterning extracellular matrix proteins on electrospun fibrous substrate promote human mesenchymal stem cell differentiation toward neurogenic lineage.* ACS Applied Materials & Interfaces, 2016. **8**(1): p. 563-573.

50. Li, Q., et al., *Differential and interactive effects of substrate topography and chemistry on human mesenchymal stem cell gene expression.* International Journal of Molecular Sciences, 2018. **19**(8).

51. Truskett, V.N. and M.P. Watts, *Trends in imprint lithography for biological applications.* Trends in Biotechnology, 2006. **24**(7): p. 312-7.

52. Nikkhah, M., et al., *Engineering microscale topographies to control the cell-substrate interface.* Biomaterials, 2012. **33**(21): p. 5230--5246.

53. Wu, Y.-n., et al., *Substrate topography determines the fate of chondrogenesis from human mesenchymal stem cells resulting in specific cartilage phenotype formation.* Nanomedicine, 2014. **10**(7): p. 1507--1516.

54. Abagnale, G., et al., *Surface topography enhances differentiation of mesenchymal stem cells towards osteogenic and adipogenic lineages.* Biomaterials, 2015. **61**: p. 316-326.

55. Tong, W.Y., et al., *Functional replication of the tendon tissue microenvironment by a bioimprinted substrate and the support of tenocytic differentiation of mesenchymal stem cells.* Biomaterials, 2012. **33**(31): p. 7686--7698.

56. Ankam, S., et al., *Substrate topography and size determine the fate of human embryonic stem cells to neuronal or glial lineage.* Acta Biomaterialia, 2013. **9**(1): p. 4535-4545.

57. You, R., et al., *Response of filopodia and lamellipodia to surface topography on micropatterned silk fibroin films.* Journal of Biomedical Materials Research Part A, 2014. **102**(12): p. 4206-4212.

58. McMurray, R.J., et al., *Surface topography regulates wnt signaling through control of primary cilia structure in mesenchymal stem cells.* Scientific Reports, 2013. **3**: p. 3545.

59. McMurray, R.J., et al., *Nanoscale surfaces for the long-term maintenance of mesenchymal stem cell phenotype and multipotency.* Nature Materials, 2011. **10**(8): p. 637-44.

60. Griffin, M.F., et al., *Control of stem cell fate by engineering their micro and nanoenvironment.* World Journal of Stem Cells, 2015. **7**(1): p. 37-50.

61. Cipriano, A.F., et al., *Bone marrow stromal cell adhesion and morphology on micro- and sub-micropatterned titanium.* Journal of Biomedical Nanotechnology, 2014. **10**(4): p. 660-668.

62. Zhang, X., et al., *Synergetic topography and chemistry cues guiding osteogenic differentiation in bone marrow stromal cells through ERK1/2 and p38 MAPK signaling pathway.* Biomaterials Science, 2018. **6**: p. 418-430.

63. Wang, J., et al., *Nanostructured titanium regulates osseointegration via influencing macrophage polarization in the osteogenic environment.* International Journal of Nanomedicine, 2018. **13**: p. 4029-4043.

64. Silverwood, R.K., et al., *Analysis of osteoclastogenesis/osteoblastogenesis on nanotopographical titania surfaces.* Advanced Healthcare Materials, 2016. **5**(8): p. 947-55.

65. Wang, J.R., et al., *Nanotopology potentiates growth hormone signalling and osteogenesis of mesenchymal stem cells.* Growth Hormone & IGF Research, 2014. **24**(6): p. 245-50.

66. Liu, X., et al., *Subcellular cell geometry on micropillars regulates stem cell differentiation.* Biomaterials, 2016. **111**: p. 27-39.

67. Wu, Y.N., et al., *Substrate topography determines the fate of chondrogenesis from human mesenchymal stem cells resulting in specific cartilage phenotype formation.* Nanomedicine, 2014. **10**(7): p. 1507-16.

68. Wu, Y., et al., *The Combined Effect of Substrate Stiffness and Surface Topography on Chondrogenic Differentiation of Mesenchymal Stem Cells.* Tissue Eng Part A, 2017. **23**(1-2): p. 43-54.

69. Roger, Y., et al., *Grid-like surface structures in thermoplastic polyurethane induce anti-inflammatory and anti-fibrotic processes in bone marrow-derived mesenchymal stem cells.* Colloids and Surfaces B: Biointerfaces, 2016. **148**: p. 104-115.

70. Klymov, A., et al., *Bone marrow-derived mesenchymal cells feature selective migration behavior on submicro- and nano-dimensional multi-patterned substrates.* Acta Biomaterialia, 2015. **16**: p. 117-25.

71. Hulshof, F.F.B., et al., *Mining for osteogenic surface topographies: In silico design to in vivo osseo-integration.* Biomaterials, 2017. **137**: p. 49-60.

72. Leuning, D.G., et al., *The cytokine secretion profile of mesenchymal stromal cells is determined by surface structure of the microenvironment.* Scientific Reports, 2018. **8**(1): p. 7716.

73. English, A., et al., *Substrate topography: a valuable in vitro tool, but a clinical red herring for in vivo tenogenesis.* Acta Biomaterialia, 2015. **27**: p. 3-12.

74. Azeem, A., et al., *The influence of anisotropic nano-to micro-topography on in vitro and in vivo osteogenesis.* Nanomedicine, 2015. **10**(5): p. 693-711.

75. English, A., et al., *Data on in vitro and in vivo cell orientation on substrates with different topographies.* Data in Brief, 2015. **5**: p. 379-382.

76. Wozniak, M.A. and C.S. Chen, *Mechanotransduction in development: a growing role for contractility.* Nature Reviews Molecular Cell Biology, 2009. **10**(1): p. 34-43.

77. Wang, H.B., M. Dembo, and Y.L. Wang, *Substrate flexibility regulates growth and apoptosis of normal but not transformed cells.* American Journal of Physiology: Cell Physiology, 2000. **279**(5): p. C1345-50.

78. Wells, R.G., *The role of matrix stiffness in regulating cell behavior.* Hepatology, 2008. **47**(4): p. 1394-400.

79. Jaalouk, D.E. and J. Lammerding, *Mechanotransduction gone awry.* Nature Reviews Molecular Cell Biology, 2009. **10**(1): p. 63-73.

80. Kubow, K.E., et al., *Mechanical forces regulate the interactions of fibronectin and collagen I in extracellular matrix.* Nature Communications, 2015. **6**: p. 8026.

81. Mitragotri, S. and J. Lahann, *Physical approaches to biomaterial design.* Nature Materials, 2009. **8**(1): p. 15-23.

82. Wozniak, M.A., et al., *Focal adhesion regulation of cell behavior.* Biochimica et Biophysica Acta, 2004. **1692**(2-3): p. 103-19.

83. Humphrey, J.D., E.R. Dufresne, and M.A. Schwartz, *Mechanotransduction and extracellular matrix homeostasis.* Nature Reviews Molecular Cell Biology, 2014. **15**(12): p. 802-12.

84. Pelham, R.J., Jr. and Y. Wang, *Cell locomotion and focal adhesions are regulated by substrate flexibility.* Proceedings of the National Academy of Science 1997. **94**(25): p. 13661-5.

85. Schwartz, M.A., *Integrins and extracellular matrix in mechanotransduction.* Cold Spring Harbor Perspectives om Biology, 2010. **2**(12): p. a005066.

86. Solon, J., et al., *Fibroblast adaptation and stiffness matching to soft elastic substrates.* Biophysical Journal, 2007. **93**(12): p. 4453-61.

87. Isenberg, B.C., et al., *Vascular smooth muscle cell durotaxis depends on substrate stiffness gradient strength.* Biophysical Journal, 2009. **97**(5): p. 1313-22.

88. Engler, A.J., et al., *Matrix elasticity directs stem cell lineage specification.* Cell, 2006. **126**(4): p. 677-89.

89. Dupont, S., et al., *Role of YAP/TAZ in mechanotransduction.* Nature, 2011. **474**(7350): p. 179-83.

90. Sun, M., et al., *Extracellular matrix stiffness controls osteogenic differentiation of mesenchymal stem cells mediated by integrin alpha5.* Stem Cell Research & Therapy, 2018. **9**(1): p. 52.

91. Rowlands, A.S., P.A. George, and J.J. Cooper-White, *Directing osteogenic and myogenic differentiation of MSCs: interplay of stiffness and adhesive ligand presentation.* American Journal of Physiology: Cell Physiology, 2008. **295**(4): p. C1037-44.

92. Park, J.S., et al., *The effect of matrix stiffness on the differentiation of mesenchymal stem cells in response to TGF-beta.* Biomaterials, 2011. **32**(16): p. 3921-30.

93. Kurpinski, K., et al., *Transforming growth factor-beta and notch signaling mediate stem cell differentiation into smooth muscle cells.* Stem Cells, 2010. **28**(4): p. 734-42.

94. Wang, D., et al., *Proteomic profiling of bone marrow mesenchymal stem cells upon transforming growth factor beta1 stimulation.* Journal of Biological Chemistry, 2004. **279**(42): p. 43725-34.

95. Williams, C.G., et al., *In vitro chondrogenesis of bone marrow-derived mesenchymal stem cells in a photopolymerizing hydrogel.* Tissue Engineering, 2003. **9**(4): p. 679-88.

96. Hebner, C., V.M. Weaver, and J. Debnath, *Modeling morphogenesis and oncogenesis in three-dimensional breast epithelial cultures.* Annual Review of Pathology, 2008. **3**: p. 313-39.

97. Gasiorowski, J.Z., C.J. Murphy, and P.F. Nealey, *Biophysical cues and cell behavior: the big impact of little things.* Annual Reviews in Biomedical Engineering, 2013. **15**: p. 155-76.

98. Trappmann, B., et al., *Extracellular-matrix tethering regulates stem-cell fate.* Nature Materials, 2012. **11**(7): p. 642-9.

99. King, D.J. and R.R. Noss, *Toxicity of polyacrylamide and acrylamide monomer.* Reviews on Environmental Health, 1989. **8**(1-4): p. 3-16.

100. Lyu, S. and D. Untereker, *Degradability of polymers for implantable biomedical devices.* International Journal of Molecular Sciences, 2009. **10**(9): p. 4033-65.

101. Lv, H., et al., *Biomaterial stiffness determines stem cell fate.* Life Sciences, 2017. **178**: p. 42-48.

102. McKee, C.T., et al., *Indentation versus tensile measurements of Young's modulus for soft biological tissues.* Tissue Engineering, 2011. **17**(3): p. 155-64.

103. Soofi, S.S., et al., *The elastic modulus of Matrigel as determined by atomic force microscopy.* Journal of Structural Biology, 2009. **167**(3): p. 216-9.

104. Vichare, S., S. Sen, and M.M. Inamdar, *Cellular mechanoadaptation to substrate mechanical properties: contributions of substrate stiffness and thickness to cell stiffness measurements using AFM.* Soft Matter, 2014. **10**(8): p. 1174-81.

105. Bara, J.J., et al., *Concise review: Bone marrow-derived mesenchymal stem cells change phenotype following in vitro culture: implications for basic research and the clinic.* Stem Cells, 2014. **32**(7): p. 1713-1723.

106. Wang, J.H. and B. Li, *Mechanics rules cell biology.* Sports Medicine, Arthroscopy, Rehabiliation, Therapy & Technology, 2010. **2**: p. 16.

107. Peroglio, M., et al., *Relevance of bioreactors and whole tissue cultures for the translation of new therapies to humans.* Journal of Orthopaedic Research, 2018. **36**(1): p. 10-21.

108. Ingber, D.E., *Cellular mechanotransduction: putting all the pieces together again.* FASEB Journal, 2006. **20**(7): p. 811-827.

109. Janmey, P.A. and C.A. McCulloch, *Cell mechanics: Integrating cell responses to mechanical stimuli.* Annual Review of Biomedical Engineering, 2007. **9**(1): p. 1-34.

110. Humphrey, J.D., E.R. Dufresne, and M.A. Schwartz, *Mechanotransduction and extracellular matrix homeostasis.* Nature Reviews Molecular Cell Biology, 2014. **15**: p. 802.

111. Vining, K.H. and D.J. Mooney, *Mechanical forces direct stem cell behaviour in development and regeneration.* Nature Reviews Molecular Cell Biology, 2017. **18**(12): p. 728-742.

112. Engelmayr, G.C., Jr., et al., *Cyclic flexure and laminar flow synergistically accelerate mesenchymal stem cell-mediated engineered tissue formation: Implications for engineered heart valve tissues.* Biomaterials, 2006. **27**(36): p. 6083-95.

113. Grottkau, B.E., et al., *Comparison of effects of mechanical stretching on osteogenic potential of ASCs and BMSCs.* Bone Research, 2013. **1**: p. 282.

114. Chen, X., et al., *Mechanical stretch-induced osteogenic differentiation of human jaw bone marrow mesenchymal stem cells (hJBMMSCs) via inhibition of the NF-κB pathway.* Cell Death & Disease, 2018. **9**(2): p. 207.

115. van Eijk, F., et al., *The effect of timing of mechanical stimulation on proliferation and differentiation of goat bone marrow stem cells cultured on braided PLGA scaffolds.* Tissue Engineering, 2008. **14**(8): p. 1425-33.

116. Nam, H.Y., et al., *The proliferation and tenogenic differentiation potential of bone marrow-derived mesenchymal stromal cell are influenced by specific uniaxial cyclic tensile loading conditions.* Biomechanics and Modelling in Mechanobiology, 2015. **14**(3): p. 649-63.

117. Nam, H.Y., et al., *Fate of tenogenic differentiation potential of human bone marrow stromal cells by uniaxial stretching affected by stretch-activated calcium channel agonist gadolinium.* PLoS ONE, 2017. **12**(6): p. e0178117.

118. Morita, Y., et al., *Determination of optimal cyclic uniaxial stretches for stem cell-to-tenocyte differentiation under a wide range of mechanical stretch conditions by evaluating gene expression and protein synthesis levels.* Acta Bioengineering and Biomechanics, 2013. **15**(3): p. 71-9.

119. Kuo, C.K. and R.S. Tuan, *Mechanoactive tenogenic differentiation of human mesenchymal stem cells.* Tissue Engineering, 2008. **14**(10): p. 1615-27.

120. Graceffa, V., et al., *Chasing Chimeras–The Elusive Stable Chondrogenic Phenotype.* Biomaterials, 2018.

121. Grad, S., et al., *Physical stimulation of chondrogenic cells in vitro: A review*. Clinical Orthopaedics and Related Research, 2011. **469**(10): p. 2764-2772.

122. Mauck, R.L., et al., *Regulation of cartilaginous ECM gene transcription by chondrocytes and MSCs in 3D culture in response to dynamic loading.* Biomechanics and Modelling in Mechanobiology, 2007. **6**(1-2): p. 113-25.

123. Mouw, J.K., et al., *Dynamic compression regulates the expression and synthesis of chondrocyte-specific matrix molecules in bone marrow stromal cells.* Stem Cells, 2007. **25**(3): p. 655-63.

124. Miyanishi, K., et al., *Dose- and time-dependent effects of cyclic hydrostatic pressure on transforming growth factor-beta3-induced chondrogenesis by adult human mesenchymal stem cells in vitro.* Tissue Engineering, 2006. **12**(8): p. 2253-62.

125. Li, Z., et al., *Chondrogenesis of human bone marrow mesenchymal stem cells in fibrin-polyurethane composites is modulated by frequency and amplitude of dynamic compression and shear stress.* Tissue Engineering, 2010. **16**(2): p. 575-84.

126. Haghighipour, N., et al., *Differential effects of cyclic uniaxial stretch on human mesenchymal stem cell into skeletal muscle cell.* Cell Biology International, 2012. **36**(7): p. 669-75.

127. Ghazanfari, S., M. Tafazzoli-Shadpour, and M.A. Shokrgozar, *Effects of cyclic stretch on proliferation of mesenchymal stem cells and their differentiation to smooth muscle cells.* Biochemical and Biophysical Research Communications, 2009. **388**(3): p. 601-605.

128. Zhao, J., et al., *Bioreactors for tissue engineering: An update.* Biochemical Engineering Journal, 2016. **109**: p. 268-281.

129. Bhatia, S.N. and D.E. Ingber, *Microfluidic organs-on-chips.* Nature Biotechnology, 2014. **32**: p. 760-772.

130. Guenat, O.T. and F. Berthiaume, *Incorporating mechanical strain in organs-on-a-chip: Lung and skin.* Biomicrofluidics, 2018. **12**(4): p. 042207.

131. Geraili, A., et al., *Controlling differentiation of stem cells for developing personalized organ-on-chip platforms.* Advanced Healthcare Materials, 2018. **7**(2): p. 1700426.

132. Simon, M.C. and B. Keith, *The role of oxygen availability in embryonic development and stem cell function.* Nature Reviews Molecular Cell Biology, 2008. **9**(4): p. 285-96.

133. Brahimi-Horn, M.C. and J. Pouyssegur, *Oxygen, a source of life and stress.* FEBS Letters, 2007. **581**(19): p. 3582-3591.

134. Dellatore, S.M., A.S. Garcia, and W.M. Miller, *Mimicking stem cell niches to increase stem cell expansion.* Current Opinion on Biotechnology, 2008. **19**(5): p. 534-540.

135. Chow, D.C., et al., *Modeling pO(2) distributions in the bone marrow hematopoietic compartment. II. Modified Kroghian models.* Biophysical Journal, 2001. **81**(2): p. 685-696.

136. Mohyeldin, A., T. Garzon-Muvdi, and A. Quinones-Hinojosa, *Oxygen in stem cell biology: a critical component of the stem cell niche.* Cell Stem Cell, 2010. **7**(2): p. 150-61.

137. Hubbi, M.E. and G.L. Semenza, *Regulation of cell proliferation by hypoxia-inducible factors.* American Journal of Physiology - Cell Physiology, 2015. **309**(12): p. C775-C782.

138. Das, R., et al., *The role of hypoxia in bone marrow-derived mesenchymal stem cells: considerations for regenerative medicine approaches.* Tissue Engineering, 2010. **16**(2): p. 159-168.

139. Tsai, C.C., et al., *Hypoxia inhibits senescence and maintains mesenchymal stem cell properties through down-regulation of E2A-p21 by HIF-TWIST.* Blood, 2011. **117**(2): p. 459-69.

140. Zhang, J., et al., *Hypoxic culture enhances the expansion of rat bone marrow-derived mesenchymal stem cells via the regulatory pathways of cell division and apoptosis.* In Vitro Cellular & Developmental Biology - Animal, 2018.

141. Kim, D.S., et al., *Effect of low oxygen tension on the biological characteristics of human bone marrow mesenchymal stem cells.* Cell Stress Chaperones, 2016. **21**(6): p. 1089-1099.

142. Lee, J.S., et al., *Enhancing proliferation and optimizing the culture condition for human bone marrow stromal cells using hypoxia and fibroblast growth factor-2.* Stem Cell Research, 2018. **28**: p. 87-95.

143. Burian, E., et al., *Effect of hypoxia on the proliferation of porcine bone marrow-derived mesenchymal stem cells and adipose-derived mesenchymal stem cells in 2- and 3-dimensional culture.* Journal of Cranio-maxillo-facial Surgery, 2017. **45**(3): p. 414-419.

144. Yu, Y., et al., *Stem cell homing-based tissue engineering using bioactive materials.* Frontiers of Materials Science, 2017. **11**(2): p. 93-105.

145. Hung, S.-C., et al., *Short-term exposure of multipotent stromal cells to low oxygen increases their expression of CX3CR1 and CXCR4 and their engraftment in vivo.* PLoS ONE, 2007. **2**(5): p. e416.

146. Yu, X., et al., *CoCl2 , a mimic of hypoxia, enhances bone marrow mesenchymal stem cells migration and osteogenic differentiation via STAT3 signaling pathway.* Cell Biology International, 2018. **42**(10): p. 1321-1329.

147. Choi, J.H., et al., *Hypoxia inducible factor-1alpha regulates the migration of bone marrow mesenchymal stem cells via integrin alpha 4.* Stem Cells International, 2016. **2016**: p. 7932185.

148. Jin, Y., et al., *Mesenchymal stem cells cultured under hypoxia escape from senescence via down-regulation of p16 and extracellular signal regulated kinase.* Biochemical and Biophysical Research Communications, 2010. **391**(3): p. 1471-6.

149. Basciano, L., et al., *Long term culture of mesenchymal stem cells in hypoxia promotes a genetic program maintaining their undifferentiated and multipotent status.* BMC Cell Biology, 2011. **12**: p. 12.

150. Satyam, A., et al., *Macromolecular crowding meets tissue engineering by self-assembly: a paradigm shift in regenerative medicine.* Advanced Materials, 2014. **26**(19): p. 3024-34.

151. Cigognini, D., et al., *Macromolecular crowding meets oxygen tension in human mesenchymal stem cell culture - A step closer to physiologically relevant in vitro organogenesis.* Scientific Reports, 2016. **6**: p. 30746.

152. Adesida, A.B., A. Mulet-Sierra, and N.M. Jomha, *Hypoxia mediated isolation and expansion enhances the chondrogenic capacity of bone marrow mesenchymal stromal cells.* Stem Cell Research & Therapy, 2012. **3**(2): p. 9-20.

153. Bornes, T.D., et al., *Hypoxic culture of bone marrow-derived mesenchymal stromal stem cells differentially enhances in vitro chondrogenesis within cell-seeded collagen and hyaluronic acid porous scaffolds.* Stem Cell Research & Therapy, 2015. **6**: p. 84.

154. Rodenas-Rochina, J., et al., *Influence of oxygen levels on chondrogenesis of porcine mesenchymal stem cells cultured in polycaprolactone scaffolds.* Journal of Biomedical Materials Research Part A, 2017. **105**(6): p. 1684-1691.

155. Zscharnack, M., et al., *Low oxygen expansion improves subsequent chondrogenesis of ovine bone-marrow-derived mesenchymal stem cells in collagen type I hydrogel.* Cells Tissues Organs, 2009. **190**(2): p. 81-93.

156. Kanichai, M., et al., *Hypoxia promotes chondrogenesis in rat mesenchymal stem cells: a role for AKT and hypoxia-inducible factor (HIF)-1alpha.* Journal of Cell Physiology, 2008. **216**(3): p. 708-15.

157. Branly, T., et al., *Characterization and use of equine bone marrow mesenchymal stem cells in equine cartilage engineering. Study of their hyaline cartilage forming potential when cultured under hypoxia within a biomaterial in the presence of BMP-2 and TGF-ss1.* Stem Cell Reviews and Reports, 2017. **13**(5): p. 611-630.

158. Lee, J.S., et al., *Human bone marrow stem cells cultured under hypoxic conditions present altered characteristics and enhanced in vivo tissue regeneration.* Bone, 2015. **78**: p. 34-45.

159. Sheehy, E.J., C.T. Buckley, and D.J. Kelly, *Oxygen tension regulates the osteogenic, chondrogenic and endochondral phenotype of bone marrow derived mesenchymal stem cells.* Biochemical and Biophysical Research Communications, 2012. **417**(1): p. 305-10.

160. Zhou, Y., et al., *Hypoxia induces osteogenic/angiogenic responses of bone marrow-derived mesenchymal stromal cells seeded on bone-derived scaffolds via ERK1/2 and p38 pathways.* Biotechnology and Bioengineering, 2013. **110**(6): p. 1794-804.

161. Ho, S.S., et al., *Hypoxic preconditioning of mesenchymal stem cells with subsequent spheroid formation accelerates repair of segmental bone defects.* Stem Cells, 2018. **36**(9): p. 1393-1403.

162. Holzwarth, C., et al., *Low physiologic oxygen tensions reduce proliferation and differentiation of human multipotent mesenchymal stromal cells.* BMC Cell Biology, 2010. **11**: p. 11.

163. Fan, L., et al., *Low oxygen tension enhances osteogenic potential of bone marrow-derived mesenchymal stem cells with osteonecrosis-related functional impairment.* Stem Cells International, 2015. **2015**: p. 950312.

164. Liu, C., et al., *Endothelial differentiation of bone marrow mesenchyme stem cells applicable to hypoxia and increased migration through Akt and NFkappaB signals.* Stem Cell Research & Therapy, 2017. **8**(1): p. 29.

165. Mung, K.L., et al., *Rapid and efficient generation of neural progenitors from adult bone marrow stromal cells by hypoxic preconditioning.* Stem Cell Research & Therapy, 2016. **7**(1): p. 146.

166. Huang, T.F., et al., *Mesenchymal stem cells from a hypoxic culture improve and engraft Achilles tendon repair.* American Journal of Sports Medicine, 2013. **41**(5): p. 1117-25.

167. Paschos, N.K., et al., *Advances in tissue engineering through stem cell-based co-culture.* Journal of Tissue Engineering and Regenerative Medicine, 2015. **9**(5): p. 488-503.

168. Park, H., et al., *Microengineered platforms for co-cultured mesenchymal stem cells towards vascularized bone tissue engineering.* Tissue Engineering and Regenerative Medicine, 2016. **13**(5): p. 465-474.

169. Caplan, A.I., *Mesenchymal stem cells.* Journal of Orthopaedic Research, 1991. **9**(5): p. 641-650.

170. Sun, J., et al., *Role of bone morphogenetic protein-2 in osteogenic differentiation of mesenchymal stem cells.* Molecular Medicine Reports, 2015. **12**(3): p. 4230-4237.

171. Guerrero, J., et al., *Cell interactions between human progenitor-derived endothelial cells and human mesenchymal stem cells in a three-dimensional macroporous polysaccharide-based scaffold promote osteogenesis.* Acta Biomaterialia, 2013. **9**(9): p. 8200-8213.

172. Zhou, J., et al., *The repair of large segmental bone defects in the rabbit with vascularized tissue engineered bone.* Biomaterials, 2010. **31**(6): p. 1171-1179.

173. Liu, Y.C., et al., *Contrasting effects of vasculogenic induction upon biaxial bioreactor stimulation of mesenchymal stem cells and endothelial progenitor cells cocultures in three-dimensional scaffolds under in vitro and in vivo paradigms for vascularized bone tissue engineering.* Tissue Engineering, 2013. **19**(7-8): p. 893-904.

174. Yoo, J.U., et al., *The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells.* Journal of Bone and Joint Surgery - American Volume, 1998. **80A**(12): p. 1745-1757.

175. Tsuchiya, K., et al., *The effect of coculture of chondrocytes with mesenchymal stem cells on their cartilaginous phenotype in vitro.* Materials Science & Engineering C-Biomimetic and Supramolecular Systems, 2004. **24**(3): p. 391-396.

176. Acharya, C., et al., *Enhanced chondrocyte proliferation and mesenchymal stromal cells chondrogenesis in coculture pellets mediate improved cartilage formation.* Journal of Cellular Physiology, 2012. **227**(1): p. 88-97.

177. Meretoja, V.V., et al., *Enhanced chondrogenesis in co-cultures with articular chondrocytes and mesenchymal stem cells.* Biomaterials, 2012. **33**(27): p. 6362-6369.

178. Chen, J.W., et al., *In vivo chondrogenesis of adult bone-marrow-derived autologous mesenchymal stem cells.* Cell and Tissue Research, 2005. **319**(3): p. 429-438.

179. Matthies, N.F., et al., *Matrix formation is enhanced in co-cultures of human meniscus cells with bone marrow stromal cells.* Journal of Tissue Engineering and Regenerative Medicine, 2013. **7**(12): p. 965-973.

180. Khurshid, M., et al., *Osteoarthritic human chondrocytes proliferate in 3D co-culture with mesenchymal stem cells in suspension bioreactors.* Journal of Tissue Engineering and Regenerative Medicine, 2018. **12**(3): p. E1418-E1432.

181. Canseco, J.A., et al., *Effect on ligament marker expression by direct-contact co-culture of mesenchymal stem cells and anterior cruciate ligament cells.* Tissue Engineering, 2012. **18**(23-24): p. 2549-2558.

182. Lee, I.C., et al., *The differentiation of mesenchymal stem cells by mechanical stress or/and co-culture system.* Biochemical and Biophysical Research Communications, 2007. **352**(1): p. 147-152.

183. Song, F.L., et al., *Mechanical loading improves tendon bone healing in a rabbit anterior cruciate ligament reconstruction model by promoting proliferation and matrix formation of mesenchymal stem cells and tendon cells.* Cellular Physiology and Biochemistry, 2017. **41**(3): p. 875-889.

184. Zhang, L., et al., *Time-related changes in expression of collagen types I and III and of tenascin-C in rat bone mesenchymal stem cells under co-culture with ligament fibroblasts or uniaxial stretching.* Cell and Tissue Research, 2008. **332**(1): p. 101-109.

185. Zhao, B., et al., *Tenascin-C expression and its associated pathway in BMSCs following co-culture with mechanically stretched ligament fibroblasts.* Molecular Medicine Reports, 2017. **15**(5): p. 2465-2472.

186. Fan, H.B., et al., *Enhanced differentiation of mesenchymal stem cells co-cultured with ligament fibroblasts on gelatin/silk fibroin hybrid scaffold.* Biomaterials, 2008. **29**(8): p. 1017-1027.

187. Wu, T.Y., et al., *The use of cocultured mesenchymal stem cells with tendon-derived stem cells as a better cell source for tendon repair.* Tissue Engineering, 2016. **22**(19-20): p. 1229-1240.

188. Luo, Q., et al., *Indirect co-culture with tenocytes promotes proliferation and mRNA expression of tendon/ligament related genes in rat bone marrow mesenchymal stem cells.* Cytotechnology, 2009. **61**(1-2): p. 1-10.

189. Lovati, A.B., et al., *Tenogenic differentiation of equine mesenchymal progenitor cells under indirect co-culture.* International Journal of Artificial Organs, 2012. **35**(11): p. 996-1005.

190. Valarmathi, M.T., et al., *A 3-D cardiac muscle construct for exploring adult marrow stem cell based myocardial regeneration.* Biomaterials, 2010. **31**(12): p. 3185-3200.

191. Popov, B.V., et al., *Lung epithelial cells induce endodermal differentiation in mouse mesenchymal bone marrow stem cells by paracrine mechanism.* Tissue Engineering, 2007. **13**(10): p. 2441-U1.

192. van Haaften, T., et al., *Airway delivery of mesenchymal stem cells prevents arrested alveolar growth in neonatal lung injury in rats.* American Journal of Respiratory and Critical Care Medicine, 2009. **180**(11): p. 1131-1142.

193. Imberti, B., et al., *Insulin-like growth factor-1 sustains stem cell-mediated renal repair.* Journal of the American Society of Nephrology, 2007. **18**(11): p. 2921-2928.

194. Plotnikov, E.Y., et al., *Cytoplasm and organelle transfer between mesenchymal multipotent stromal cells and renal tubular cells in co-culture.* Experimental Cell Research, 2010. **316**(15): p. 2447-2455.

195. Mizuguchi, T., et al., *Enhanced proliferation and differentiation of rat hepatocytes cultured with bone marrow stromal cells.* Journal of Cellular Physiology, 2001. **189**(1): p. 106-119.

196. Gu, J.Y., et al., *Heterotypic interactions in the preservation of morphology and functionality of porcine hepatocytes by bone marrow mesenchymal stem cells in vitro.* Journal of Cellular Physiology, 2009. **219**(1): p. 100-108.

197. Yagi, H., et al., *Long-term superior performance of a stem cell/hepatocyte device for the treatment of acute liver failure.* Tissue Engineering, 2009. **15**(11): p. 3377-3388.

198. Sitanggang, E.J., et al., *Bone marrow stem cells anti-liver fibrosis potency: Inhibition of hepatic stellate cells activity and extracellular matrix deposition.* International Journal of Stem Cells, 2017. **10**(1): p. 69-75.

199. Shologu, N., et al., *Recreating complex pathophysiologies in vitro with extracellular matrix surrogates for anticancer therapeutics screening.* Drug Discovery Today, 2016. **21**(9): p. 1521-1531.

200. Kumar, P., et al., *Accelerated development of supramolecular corneal stromal-like assemblies from corneal fibroblasts in the presence of macromolecular crowders.* Tissue Engineering, 2015. **21**(7): p. 660-670.

201. Peng, Y., et al., *Human fibroblast matrices bio-assembled under macromolecular crowding support stable propagation of human embryonic stem cells.* Journal of Tissue Engineering and Regenerative Medicine, 2012. **6**(10): p. e74-e86.